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Phenotypic plasticity in response to environmental heterogeneity contributes to fluctuating asymmetry in plants: first empirical evidence

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Abstract

Fluctuating asymmetry (FA) is widely used to quantify developmental instability (DI) in ecological and evolutionary studies. It has long been recognized that FA may not exclusively originate from DI for sessile organisms such as plants, because phenotypic plasticity in response to heterogeneities in the environment might also produce FA. This study provides the first empirical evidence for this hypothesis. We reasoned that solar irradiance, which is greater on the southern side than on the northern side of plants growing in the temperate zone of the northern hemisphere, would cause systematic morphological differences and asymmetry associated with the orientation of plant parts. We used geometric morphometrics to characterize the size and shape of flower parts in *Iris pumila* grown in a common garden. The size of floral organs was not significantly affected by orientation. Shape and particularly its asymmetric component differed significantly according to orientation for three different floral parts. Orientation accounted for 10.4% of the total shape asymmetry within flowers in the falls, for 11.4% in the standards, and for 2.2% in the style branches. This indicates that phenotypic plasticity in response to a directed environmental factor, most likely solar irradiance, contributes to FA of flowers under natural conditions. That FA partly results from phenotypic plasticity and not just from DI needs to be considered by studies of FA in plants and other sessile organisms.

Keywords: developmental instability, fluctuating asymmetry, geometric morphometrics, *Iris pumila*, phenotypic plasticity, shape

Introduction

Fluctuating asymmetry (FA) is a kind of phenotypic variation that manifests itself as the variable left–right difference in size or shape of bilaterally symmetric structures or as the variation among repeated parts in structures with complex symmetry (Palmer & Strobeck, 1986, 2003; Graham *et al.*, 2010; Savriama & Klingenberg, 2011; Klingenberg, 2015). FA is widely used in ecology and evolutionary biology as an easily measurable indicator of environmental and genetic stress (Palmer & Strobeck, 1986, 2003; Parsons, 1992; Wilsey *et al.*, 1998; Waldmann, 2001; Tucić *et al.*, 2008; Tucić & Miljković, 2010; Raz *et al.*, 2011; Beasley *et al.*, 2013; Abeli *et al.*, 2016; Sandner & Matthies, 2017; Telhado *et al.*, 2017), individual quality (Møller, 1995; Møller & Shykoff, 1999; Cornelissen & Stiling, 2005; Frey & Bukoski, 2014), and fitness (Andalo *et al.*, 2000; Lens *et al.*, 2002; Komac & Alados, 2012). These and other studies have yielded mixed results and the whole approach of using FA as an indicator of stress or individual quality has led to considerable controversy (Palmer, 1996; Houle, 1998; Simmons *et al.*, 1999; Palmer & Hammond, 2000; Leamy & Klingenberg, 2005; Van Dongen, 2006; Debat, 2016). FA has also been widely used to investigate the developmental origin of morphological integration (Klingenberg, 2003b, 2015; Pélabon *et al.*, 2006; Zelditch *et al.*, 2009; Ivanović & Kalezić, 2010; Jamniczky & Hallgrímsson, 2011; Labonne *et al.*, 2014).

FA is considered to be the phenotypic outcome of small random irregularities in developmental processes that occur even under constant genetic and environmental conditions (Palmer, 1996; Klingenberg & Nijhout, 1999; Klingenberg, 2003a, 2015; Polak, 2003). The basic idea is that the left and the right sides of a bilaterally symmetrical organism (or of a bilaterally symmetric organ) are separate copies of a morphological structure that develop under the control of the same genome and under the same environmental conditions. If the development of

morphological structures were an entirely deterministic process, then the left and the right copies should develop as exact mirror images of each other, both exactly displaying the target phenotype specific for the genotype and environment of each individual (Nijhout & Davidowitz, 2003). In real biological systems, however, the process of development is not fully deterministic, but is affected by intrinsic developmental noise so that the realized phenotype deviates to a greater or lesser degree from the target phenotype expected under a given genotype and environmental conditions (Klingenberg, 2003a; Nijhout & Davidowitz, 2003). Because random developmental perturbations occur independently on each side, their effects are unlikely to be the same on both body sides, and the resulting differences are manifest as FA of morphological traits. Genetic and environmental effects may affect how the developmental system produces such random variation and modulates its phenotypic expression, and thus can affect the observable FA (Klingenberg & Nijhout, 1999; Klingenberg, 2003a). Applications of FA as an expression of developmental instability, regardless whether they aim to quantify the effects of environmental and genetic stress or to investigate the developmental origins of morphological integration, all make the assumption that FA originates from random developmental perturbations.

If FA is to be interpreted as the phenotypic consequence of developmental instability, a further crucial assumption is that the left and right sides of an organism or structure share the same genome and the same environment (Palmer, 1996; Klingenberg, 2003a, 2015; Nijhout & Davidowitz, 2003). Although somatic mutations have been demonstrated in many species, they appear not to contribute substantially to phenotypic variation within individuals (Herrera, 2009), so that genetic variation is unlikely to be a major contributing factor for asymmetry. For environmental variation, the usual argument is that environmental differences between sides are

small or average out over the period of development of an organism (Nijhout & Davidowitz, 2003; Klingenberg, 2015). Whereas this argument is plausible for motile organisms that move through their environment, it is unlikely to hold for sessile organisms, such as most plants, because their parts are exposed to heterogeneity in their immediate environment in a constant manner. For instance, heterogeneous shading by nearby leaves may produce persistent differences in the incident light between the left and right sides of a single leaf. If phenotypic plasticity leads to a morphological response to such environmental heterogeneity, the resulting asymmetry is a component of FA that is not due to developmental instability. In turn, this raises the question whether FA can be used as a reliable measure of stress or fitness in sessile organisms. That FA in plants and other sessile organisms may be due in part to phenotypic plasticity in response to environmental heterogeneity has been discussed in the literature as a possibility (Palmer, 1996; Nijhout & Davidowitz, 2003; Van Dongen, 2006; Klingenberg *et al.*, 2012; Savriama *et al.*, 2012; Klingenberg, 2015) but so far there is no direct evidence for this effect.

To obtain such evidence, it seems the most elegant approach would be an experiment in which plants are grown in a completely homogeneous environment, and morphological asymmetry is measured to examine whether it is reduced by comparison to plants grown under natural conditions. Eliminating heterogeneity of environmental factors is feasible for some factors (e.g. Koethe *et al.*, 2017), but not for others. For instance, it is impossible to ensure that plant parts experience perfectly homogeneous lighting conditions because different parts of the same plant inevitably shade each other to some degree. Therefore, it is not feasible to conduct an experiment that would completely preclude FA due to plasticity. An alternative is the opposite experimental approach, in which persistent localized heterogeneity is produced for some

environmental factor such as light, temperature or humidity, and the resulting effect on morphological asymmetry is recorded. For instance, previous experiments have shown that completely covering half of a leaf can produce measurable asymmetry (Freeman *et al.*, 2003). This approach raises the question, however, whether such experiments are realistic. Experimental manipulations tend to be relatively large, in order to overcome possible procedural imprecision and artifacts, but it is not clear whether the less drastic heterogeneities that occur in natural environments are also sufficient to cause asymmetry. Such experiments can establish that a particular environmental factor has the potential to affect asymmetry, but they cannot indicate whether this factor has a sufficiently strong effect under natural conditions or whether other factors might not be equally or more important. As a consequence, this approach is able to demonstrate that plasticity in response to environmental heterogeneity can produce asymmetry in principle, but it cannot tell whether this actually occurs in nature. Therefore, rather than conducting experimental manipulations, it seems preferable to employ a natural source of environmental heterogeneity.

For testing the hypothesis that phenotypic plasticity contributes to plant FA in nature, it is helpful to focus on a natural component of environmental heterogeneity that forms a consistent gradient and thus affects many plants in the same way, so that the effect can be demonstrated using statistical approaches. Plant parts with different orientations experience the gradient at different angles in relation to their anatomical axes (Fig. 1). If phenotypic plasticity produces a response to such a gradient, parts with different orientation will differ from each other in a manner that is systematically linked to their orientations. In other words, one would expect differences in the average morphology of parts according to their orientation relative to the gradient, which is fairly straightforward to demonstrate. This leaves the question what

environmental gradient can be used for such an experiment. A suitable environmental factor with such a gradient is solar irradiance. Solar irradiance has profound physiological effects on plant development through both visible light and temperature (Larcher, 2003) and it is highly directional. When integrated throughout the day in locations in the temperate zone of the northern hemisphere, solar radiation is predominantly from southerly directions. Therefore, plant organs oriented toward the south receive more irradiance on average than organs oriented toward the north, and phenotypic plasticity may produce morphological differences between them. Also, for organs directed toward the east, there tends to be more irradiance from the right than from the left side, and the reverse for organs directed toward the west, so that phenotypic plasticity in response to solar irradiance may also cause individual plant organs to be asymmetric in ways that depend on their orientation (Fig. 1). Because of the effects of shading and reflection by objects in the immediate surroundings (e.g. by parts of the same plant or even the same flower), we expect that the actual distribution of incident light is more complex than a simple gradient. Nevertheless, we can expect that, even though the specific conditions experienced by each organ may be patterned irregularly, the directional nature of solar irradiance will produce a component that is itself directional, so that response elicited by phenotypic plasticity has a component that is consistent among all plants in the experiment and related to the orientation of the parts. Therefore, it is possible to use this directed component for testing the hypothesis that plasticity contributes to FA by examining whether plant organs with different compass orientations differ in the averages of their shapes and asymmetries.

This study presents the first empirical test of the hypothesis that phenotypic plasticity in response to environmental heterogeneity contributes to FA in plant organs. We investigate the floral organs of *Iris pumila*, a species that previously has been used in studies of FA using plants

153 from a common garden experiment (Tucić *et al.*, 2008, 2013; Radović *et al.*, 2017) and from
154 contrasting light habitats in the wild (Tucić & Miljković, 2010). To test the hypothesis, we use
155 the methods of geometric morphometrics (Klingenberg, 2010; Zelditch *et al.*, 2012; Adams *et*
156 *al.*, 2013) to quantify shape variation and asymmetry of three different floral organs in relation to
157 their compass orientations.

158 ## Material and Methods

159 *Study Species and Experimental Set-up*

160 *Iris pumila* L. is a rhizomatous perennial plant that is widespread in the lowlands of Central and
161 Southeast Europe (Randolph, 1955). In Serbia, the species is native to the Deliblato Sands (44˚
162 47' N, 21˚ 20' E; Gajić, 1983), where it forms round clones differing in size, depending on their
163 age (Tucić *et al.*, 1988). The species blooms in early spring, and the flowering phase lasts about
164 two to three weeks.

165 The flower of *I. pumila*, similar to other species of *Iris* (Pande & Singh, 1981), consists of four
166 trimerous whorls: two whorls of tepals, the stamens and the gynoecium, of which the petaloid
167 style branches form a conspicuous part of the flower (Fig. 2A). The bases of the tepals are united
168 to form a floral tube (Fig. 2A: FT). The outer tepals are called "falls" and are bent downwards to
169 function as a landing platform for pollinating insects (Fig. 2A: F). The inner tepals, called
170 "standards", are erect and are the flower elements that are the most visible from a distance (Fig.
171 2A: S). The stamens (Fig. 2A: Sta) are hidden below the style branches (Fig. 2A: StyB), which
172 bend over the basal part of the falls and carry the receptive stigmatic lip near their tip (Fig. 2A:
173 SL).

174 The flowers of *I. pumila* are actinomorphic, with floral organs arranged around a central axis so
175 that rotations by an angle of 120˚ separate the organs in the same whorl from each other (Fig.
176 2B). In addition to this symmetry of the flower as a whole, each of the individual flower organs
177 is bilaterally symmetrical. We take into account this complex symmetry of the flower in the
178 morphometric analyses (Savriama & Klingenberg, 2011; Klingenberg, 2015). For the whole
179 flower, we use the perspective of matching symmetry by separating the flower into individual
180 organs: the falls, standards and style branches. Asymmetry of the whole flower can be
181 characterized by the differences among the three copies of organs in each whorl. For each flower
182 organ, our analyses use the approach for bilateral object symmetry to extract symmetric and
183 asymmetry components (Klingenberg *et al.*, 2002; Klingenberg, 2015). Therefore, it is possible
184 to examine how the organs at different positions within each whorl differ in their symmetric
185 component of shape and in their shape asymmetries, both of which may be affected by exposure
186 to an environmental gradient (Fig. 1B).

187 The plants used in this study are part of a common garden experiment established in 1996 from a
188 natural population of *I. pumila* from the Deliblato Sands area. The plants were grown in clay pots
189 in an experimental garden in the grounds of the Siniša Stanković Institute for Biological
190 Research in Belgrade (44˚ 49' 2.94" N/ 20˚ 29' 15.51" E), where they still grow as mature clones
191 under common garden conditions (Manitašević Jovanović et al. 2011; Tucić et al. 2013). The
192 pots were positioned haphazardly, without any reference to the plants within them, so that the
193 orientations of the plants were effectively randomized. During the period of development of the
194 flowers used in this experiment, the pots were not moved.

195 *Collection of Samples*

196 Flowers were collected daily from 21 March to 1 April 2014, for a period starting at 11am and
197 lasting between one and two hours each day, and compass orientation was recorded for each
198 flower. For practical reasons, the orientation of flower organs was determined in relation to the
199 sun. During the sampling period, the direction of the sun at 11am was approximately from south-
200 southeast (azimuth 164.08˚ to 164.05˚ from 21 March to 29 March and 143.67˚ to 143.40˚ from
201 30 March to 1 April; the jump is because of the switch to summer time on 30 March 2014;
202 calculations using the NOAA Solar Calculator, http://www.esrl.noaa.gov/gmd/grad/solcalc/).
203 Solar noon was between 11.42am to 11.45am from 21 March to 29 March, or roughly midway
204 through the daily sampling period, and at 12.42pm from 30 March to 1 April. Overall, the
205 position of the sun approximately indicates south, more exactly so during the first nine days of
206 flower harvesting than during the last three days.

207 For each of 267 potted clones (genets), two simultaneously opened flowers were marked and
208 harvested: one with a fall oriented toward the sun and another with a standard toward the sun
209 (Fig. 2B). Because floral organs in the *Iris* flower are repeated at 120˚ intervals, this sampling
210 design resulted in a dataset with copies of each floral organ from six different orientations: 0˚
211 (toward the sun, approximately south), 120˚ and 240˚ from one flower and 60˚, 180˚ and 360˚
212 from the other flower of the same genet (Fig. 2B).

213 Immediately after harvesting, flowers were submerged in 70% ethanol and stored singly in
214 bottles until dissection. In the laboratory, every flower was cut at the end of the floral tube to
215 separate the floral organs. The falls, standards, and styles were then spread on a glass plate
216 coated with 50% glycerol. Digital images (600dpi resolution) of floral organs were recorded
217 using an optical scanner (CanoScan 5600F).

Landmark Data

To characterize the shape of floral organs, we applied the methods of geometric morphometrics, which use the relative positions of a set of landmarks to quantify morphological variation (Klingenberg, 2010; Zelditch *et al.*, 2012; Dryden & Mardia, 2016). Landmarks were digitized using tpsDig software (Rohlf, 2006). The landmark data have been deposited at DataDryad (DOI: doi:10.5061/dryad.8th5m).

For the fall, a set of 18 landmarks is used (seven pairs and four median landmarks; Fig. 3A). At the base of the fall, landmark 1 is on the central nerve, landmarks 5 and 6 are on the left and right peripheral nerves, and landmarks 7 and 8 are at the left and right margins, respectively. The tip of the fall is marked by landmark 2; landmark 3 is located at the first branching of the central nerve and landmark 4 is at the end of the beard. Landmarks 9 and 10 are on the left and right margins, at the same level as landmark 4. The remaining landmarks are distributed at equal distances on the margins between the landmarks defined before (11 and 13 between 7 and 9; 12 and 14 between 8 and 10; 15 and 17 between 2 and 9; 16 and 18 between 2 and 10).

For the standard, 19 landmarks are used (eight pairs and three median landmarks; Fig. 3B). Landmarks 1 and 2 are at the tip and a base of the central nerve. At the base of the standard, landmarks 3 and 4 are on the two peripheral nerves, while landmarks 5 and 6 are on the left and right margins. Landmarks 7 and 8 are at the points of maximal curvature where the narrow base broadens into the main blade of the standard, and landmarks 9 and 10 are the widest points of the standard. Several landmarks are equally spaced on the margin between previously defined landmarks (11 between 7 and 9; 12 between 8 and 10; 13, 15 and 17 between 2 and 9; 14, 16 and 18 between 2 and 10). Landmark 19 indicates the first branching of the central nerve.

For the style branch, 18 landmarks are used (eight pairs and two median landmarks; Fig. 3C). At the base, landmark 1is the central point, midway between the two central nerves, landmarks 3 and 4 are at the left and right central nerves, and landmarks 5 and 6 are at the left and right margins, respectively. The remaining landmarks are located on the stigma: landmark 2 is the midpoint of the apical margin of the stigma, whereas the others are arranged as pairs on the basal (landmarks 7 and 8) and apical margin of the stigmatic lip (landmarks 9–18; Fig. 3C). It was not possible to locate landmarks on the lobes at the end of the style branch because of the great variability of this region.

Morphometric analysis

As a measure of size for each floral organ, we used centroid size, the square root of the sum of squared distances of all the landmarks from their centroid (Dryden & Mardia, 2016). The differences in the sizes among organs in different orientations were tested by a one-way ANOVA. Statistical analyses of centroid size were carried out with SAS statistical software (SAS Institute Inc. 2010).

Because the floral organs were separated and flattened to collect landmark data, this study uses the framework of matching symmetry at the level of the whole flower, whereas each organ has bilateral object symmetry (Savriama & Klingenberg, 2011; Klingenberg, 2015). Accordingly, asymmetry at the level of the entire flower is characterized by the differences among the sizes and shapes of organs with different orientations. In addition, because individual flower organs are bilaterally symmetric, there are two separate components of symmetric and asymmetric shape variation for each of them, which may be differently affected by exposure to an environmental gradient under different orientations (Fig. 1B). We therefore conduct comparisons

of the flower organs with different orientations separately for the symmetric and asymmetry components of shape variation.

To extract shape information from the landmark configurations of floral organs, we used Procrustes superimpositions (Dryden & Mardia, 2016). To take into account the bilateral symmetry of floral organs, we applied the method for object symmetry, which uses the landmark configurations and their reflected and relabeled copies (Klingenberg *et al.*, 2002; Klingenberg, 2015). This method obtains the a symmetric component of shape variation by averaging the original and reflected and relabeled copies, and the asymmetric component from differences between them (Klingenberg *et al.*, 2002). Procrustes superimpositions and subsequent morphometric analyses were carried out with the MorphoJ software package (Klingenberg, 2011).

Differences among the mean shapes of floral organs according to their orientation were computed as deviations of the mean shapes for the six orientations from the overall mean shape and exaggerated 5- or 15-fold for better visibility in the diagrams. These differences were visualized as warped outline drawings, which facilitate interpretation of shape changes in their anatomical context (Klingenberg, 2013).

To assess differences in shape between floral organs with different orientations statistically, we used canonical variate analysis (CVA), a technique providing an ordination that maximizes the differences among group means relative to within-group variation (Zelditch *et al.*, 2012). CVAs were conducted separately for the symmetric and asymmetric components of shape variation of each floral organ. The variation within groups, the residual 'error' effect against which the differences among orientations are assessed in the CVAs, includes FA from developmental

284 instability, FA from phenotypic plasticity in response to environmental heterogeneity that affects
285 different flowers differently, as well as measurement error. The statistical significance of
286 pairwise differences in mean shapes was assessed with permutation test using Mahalanobis and
287 Procrustes distances (10,000 permutations per test).

288 To quantify the amount of variation for which compass orientation accounts, which is a part (but
289 not all) of the asymmetry contributed by phenotypic plasticity, we used the decomposition of
290 Procrustes sums of squares for complex matching symmetry according to formula (2) in
291 Savriama & Klingenberg (2011). We expanded the decomposition by including the additional
292 effect of flowers nested within plants. Because of the object symmetry of each floral part, we
293 computed the Procrustes sums of squares separately for the symmetric and asymmetry
294 components, and also combined as a measure of variation in the entire shape space of each
295 landmark configuration. To quantify the proportion of FA attributable to the orientation of floral
296 parts, we computed the percentages of the sums of squares of the asymmetry due to orientation
297 and the remaining asymmetry relative to the total asymmetry within flowers. In conventional
298 studies of asymmetry, without recording compass orientation of flower parts, both these
299 components of asymmetry would be considered as part of FA (i.e. no estimate of directional
300 asymmetry is available in radially symmetric flowers without a clear adaxial–abaxial direction;
301 Klingenberg, 2015). The component of asymmetry due to orientation and the residual asymmetry
302 within flowers can therefore be added up to compute the total estimate of FA that would be
303 obtained in a conventional study not recording compass orientation. The proportion of this total
304 for which orientation accounts is a lower bound for the proportion of FA due to phenotypic
305 plasticity, but is most likely an understestimate of the true proportion because it accounts only
306 for the part of environmental heterogeneity that is the same for all flowers.

Results

The mean centroid sizes of the flower organs were very nearly the same regardless of their orientations (Table 1). The ANOVAs indicated no significant differences due to orientation of falls ($F = 0.82$; df = 5, 1588; P=0.54), standards ($F = 1.39$; df = 5, 1566; P=0.22) and style branches ($F = 0.11$; df = 5, 1536; P= 0.99).

The shapes of the falls differed among orientations in subtle ways (Fig. 4). For the symmetric component of shape variation, these differences particularly affected the relative width of the base of the falls, which was especially narrow for the most southerly orientation (0˚, Fig. 4A). For the asymmetry component, the most obvious feature was the "pinwheel symmetry" of the falls—each of them is asymmetric in that the mid vein is shifted towards one side of the fall (counter-clockwise; Fig. 4B). Superimposed on this overall asymmetry, there are subtle asymmetries specific to the different orientations. The ordinations of the CVA plots provide a summary of the patterns of differences among orientations (Fig. 4C and D). For both the symmetric and asymmetry components, some confidence ellipses are clearly separated from each other, whereas some others overlap, suggesting that there were statistically significant shape differences among falls of different orientations. This finding is consistent with the distances between shape means and the results of the permutation tests (Tables S1, S2). For the symmetric component, the plot of CV scores indicated no clear pattern (Fig. 4C). For the asymmetry component, however, the sample mean shapes were arranged approximately as a ring (Fig 3D): starting at the 0˚ sample, continuing through the 60˚ sample, to the shared location of the 120˚ and 180˚ samples (not statistically different), on to the 240˚ and 300˚ samples and back to the 0˚ sample. This indicates that, for the asymmetric component of shape variation in the falls, the differences among samples for the different orientations correspond approximately to their

spatial arrangement in the flowers. Of the total shape asymmetry among falls within flowers, orientation accounted for 11.5% of asymmetry in the symmetric component, for 5.7% in the asymmetry component and for 10.4% in the combined shape components (Table 2).

For the standards, the symmetric component of variation featured differences in the relative lengths and widths of the base versus the expanded blade (Fig. 5A). As for the falls (Fig. 4A), the standards in the 0˚ position were narrowest (Fig. 5A; but note that these were not part of the same flowers because falls and standards are offset by 60˚). The asymmetric component of shape variation for the standards (Fig. 5B), as for the falls (Fig. 4B), displays clear "pinwheel" symmetry in addition to a variety of asymmetries specific to each orientation. The CVA plot for the symmetric component of variation displays no clear pattern, with some evident differences among samples but also overlap among some of them (Fig. 5C). In the CVA plot of the asymmetry component of shape variation in the standards (Fig. 5D), the mean shapes of the six samples were arranged approximately in a ring—from the 0˚ sample to the 60˚ sample, on to 120˚ and 180˚ (those are not significantly different in the permutation tests; Tables S1, S2), further on to 240˚, then 300˚ and back to the 0˚ sample. The proportion of the total asymmetry within flowers explained by orientation was 12.8% for the symmetric component, 7.3% for the asymmetry component and 11.4% for total shape variation of the standards (Table 2).

For the style branches, the symmetric component of variation featured fairly subtle differences among orientations dominated by a contrast of relative length versus width (Fig. 6A). The asymmetry component featured "pinwheel" symmetry with a clockwise displacement of the apical landmarks of the stigmatic lip relative to the more proximal landmarks and more subtle asymmetries specific to the six positions (Fig. 6B). The CVA for the symmetric component of style shape variation showed no clear pattern and extensive overlap among the confidence

intervals of the mean shapes (Fig. 6C). The permutation tests of the differences among shape averages for the different orientations provided no evidence for differences in the symmetric component of shape, whereas for the asymmetry component some significant differences were present (Tables S1, S2). For the asymmetry component of style shape, confidence ellipses for the sample means of the different positions were arranged as a ring, starting from the 0˚ sample through the 60˚ sample to the position of the 120˚ and 180˚ samples, which overlapped almost perfectly and did not differ from each other significantly, on to 240˚ through 300˚ and back to the 0˚ sample (Fig. 6D). Orientation accounted only for a minor proportion of the total asymmetry of style shape within flowers: 1.6% for the symmetric component, 3.1% for the asymmetry component and 2.2% for total shape variation (Table 2).

Discussion

The hypothesis that phenotypic plasticity in response to environmental heterogeneity contributes to FA predicts that, for plant structures exposed to a gradient from a directed environmental factor such as solar irradiance, there should be systematic differences among parts according to their orientations (Fig. 1). In agreement with this expectation, this study shows that floral organs of *I. pumila* with different orientations differ in their shapes, and particularly in their asymmetries. The effects are fairly subtle, accounting for between 1.6% and 12.8% of FA in the corresponding components of variation, but statistically significant differences exist for all three floral organs examined here. By contrast, there does not appear to be an effect on the size of floral organs.

For the symmetric component of shape variation of all three flower organs, the main feature of differences among positions was variation in the relative length versus width (Fig. 4A,

5A and 6A). The analyses revealed clear shape differences according to position for the falls and standards, but no significant differences for the style branches. It is tempting to attribute that pattern to the fact that the style branches are innermost in the developing bud and therefore might be protected from environmental effects to some extent by the other organs, but the clear effects of position on the asymmetry of the style branches (Fig. 6B and D) refute such reasoning. The CVA plots for the symmetric component (Fig. 4C, 5C and 6C) suggested no evident pattern relating either to the orientation on the flowers or to whether the organs were from the same or different flowers (orientations 0˚, 120˚ and 240˚ versus 60˚, 180˚ and 300˚).

For the asymmetry component, the most immediately striking pattern in the shape changes was the "pinwheel" symmetry of all three floral organs (Fig. 4B, 4B and 5B). It is plausible that this pattern relates to the convolute aestivation of the flower parts, where the floral organs are rolled up in the bud in a direction that is constant among flowers, as it is known across the genus *Iris* (Schoute, 1935). Superimposed on this is a subtler pattern of differences in asymmetry among the six orientations, which is most apparent from the CVA plots (Fig. 4D, 5D and 5D). For the asymmetry components of all three organs, the averages for the six orientations are arranged approximately in a ring. Although these averages do not form a perfectly regular hexagon, a relation of the asymmetry of flower organs to their spatial orientation on the flowers is clearly evident. Because the direction of CVA axes is arbitrary, it is immaterial whether the averages appear in clockwise or in counter-clockwise order and in which region of the plots each particular orientation appears (the plots can be flipped freely about their horizontal or vertical axes).

Exposure of plants to a gradient from a directed environmental factor (Fig. 1) is expected to produce a response that is the same for all plants. If there is phenotypic plasticity in response

398 to this factor, it can be assessed by recording the compass orientation of flower organs and
399 examining whether there are consistent differences between the shapes of flower organs with
400 different orientations. The differences among shape averages of flower organs with different
401 orientations, both in the symmetric and asymmetric components of shape of each organ, indicate
402 systematic asymmetries of the whole flower. Accordingly, the shape differences recorded in this
403 study are directional asymmetries, that is, systematic differences between the average shapes of
404 repeated parts within flowers (Klingenberg, 2015). Compared to other studies on plant
405 asymmetry, the present study is unique in that the compass orientations of the flower parts were
406 recorded. Previous studies have defined asymmetry in relation to plant architecture, such as the
407 adaxial–abaxial axis of flowers (Savriama *et al.*, 2012; Baranov & Gavrikov, 2013; Gardner *et*
408 *al.*, 2016) or the left-right asymmetry of leaves (Pélabon *et al.*, 2006; Chitwood *et al.*, 2012;
409 Martinez *et al.*, 2016), but did not record compass orientation of plant organs, and therefore
410 would have included asymmetries according to orientation as a component of FA. There might
411 be directional asymmetry within the flowers in relation to plant architecture in *Iris pumila* too, as
412 there is a consistent arrangement of the flower parts relative to the spathe subtending the flower
413 (pers.obs.; for another species, see Pande & Singh 1981). Any such directional asymmetry would
414 have to be subtle too, but no morphometric information of this is currently available. Because the
415 pots with plants were positioned in random orientations, however, any intrinsic asymmetry in
416 relation to the whole plant cannot be the cause for the observed systematic differences between
417 the average shapes of flower parts according to their compass orientations. Therefore, the
418 directional asymmetry according to compass orientation must be plastic response to some
419 directed environmental factor. Recording the orientation of flower parts enabled us to
420 demonstrate the effect of plasticity in response to a directed environmental factor as directional

asymmetry, because such a factor affects a large number of flowers in the same way, and therefore even subtle effects can be documented by statistical methods. This made it possible, for the first time, to show empirically that plasticity in response to environmental heterogeneity indeed contributes to morphological asymmetry in plants (Palmer, 1996; Nijhout & Davidowitz, 2003; Klingenberg *et al.*, 2012; Savriama *et al.*, 2012; Klingenberg, 2015)

The only plausible explanation for the fairly regular patterns of asymmetry (Fig. 4D, 5D and 6D) is phenotypic plasticity of the floral organs in response to a consistently directed environmental factor (Fig. 1). The most consistent irregularity in the arrangement of average shape asymmetries in the CVA plots is the partial or complete overlap and non-significant differences between the 120˚ and 180˚ orientations (Fig. 4D, 5D and 6D; Tables S1, S2). With the information at hand, we cannot offer an explanation for this irregularity. The most likely the environmental factor responsible for these effects is solar irradiance, which is known to have profound effects on physiological processes in plants through both heat and visible light (Larcher, 2003; Taiz & Zeiger, 2010). Phenotypic plasticity of plant organ shape in response to differences in irradiance has been demonstrated even within shoots (Kubínová *et al.*, 2017), and experiments have shown that floral organs can show plasticity in response to intensity and spectral composition of light (Weinig, 2002; Brock & Weinig, 2007; Kurepin *et al.*, 2016). Nevertheless, we acknowledge that other directed factors, such as geomagnetism (Maffei, 2014), cannot be ruled out on the basis of our data, but they are much less plausible as mechanisms that might account for the observed shape differences. Because *Iris* flowers grow in an upright position, asymmetry in response to gravity, which has been shown to influence asymmetry of petal positions in some *Saxifraga* species (Koethe *et al.*, 2017), also cannot be the factor responsible for the effects of compass orientation.

This demonstration that plasticity in response to environmental heterogeneity contributes to FA has substantial implications for the growing number of studies that use FA in plant parts as an indicator of developmental instability to measure the effects of environmental stresses such as pollution or unfavorable growing conditions (Kozlov *et al.*, 1996; Cornelissen & Stiling, 2010; Raz *et al.*, 2011; Baranov, 2014), to assess plant quality in plant-herbivore and plant-pollinator interactions (Møller, 1995; Cornelissen & Stiling, 2005; Anton *et al.*, 2013; Frey & Bukoski, 2014; Alves-Silva & Del-Claro, 2016), or to gauge the effects of genetic factors such as hybridization or inbreeding (Siikamäki & Lammi, 1998; Waldmann, 2001; Rao *et al.*, 2002; Albarrán-Lara *et al.*, 2010; Vaupel & Matthies, 2012; Helsen & Van Dongen, 2016; Sandner & Matthies, 2017). Because FA results not only from developmental instability, but also from plasticity in response to heterogeneity in the immediate surroundings of the plant parts, explanations of the association between FA and other factors can be ambiguous. For instance, in studies that found higher FA for leaves or flowers more exposed to sunlight than for those from more shaded positions in the same trees (Cowart & Graham, 1999; Perfectti & Camacho, 1999), there may be two alternative explanations: positions more exposed to light may be more stressful, leading to greater developmental instability and thus FA, or the greater FA may result from greater effects of plasticity in response to the sharper differences between light and shade in more exposed positions. Likewise, in comparisons of FA in plants between different environments, differences in FA might reflect greater developmental instability or more accentuated microenvironmental heterogeneity in some locations than in others. For example, observations that FA in sun-exposed habitats is greater than in shaded habitats (Tucić & Miljković, 2010; Raz *et al.*, 2011) might be explained by increased developmental instability due to light or heat stress or, alternatively, by plasticity in response to the more drastic contrasts

between the lit and shaded sides of each plant organ. Also, because FA from phenotypic plasticity simply adds to the observed asymmetry without any necessary relation to developmental instability, the additional noise it provides may contribute to the many negative results in studies attempting to correlate FA to stress, individual quality or fitness (Palmer & Strobeck, 2003; Van Dongen, 2006; Debat, 2016).

The demonstration that FA originates in part from phenotypic plasticity in response to environmental heterogeneity raises the question of how much FA is due to plasticity. Depending on which floral organ and component of shape variation is considered, orientation accounts for 1.6% to 12.8% of FA (Table 2). Because these calculations consider only aspects of local heterogeneity in environmental factors that are affecting all the flowers in the same way, but ignore all those aspects of heterogeneity that act in more irregular ways, these values are minimal estimates of how much of FA is due to phenotypic plasticity. Almost certainly, the true proportions will be greater because the environmental factors have patterns that are locally patchy and do not conform to a simple gradient, so that their effects will differ from plant to plant. To quantify how much FA actually originates from phenotypic plasticity, it would be necessary to identify all factors that might elicit phenotypic plasticity, characterize all the respective reaction norms, and measure the heterogeneity of the relevant factors in the surroundings of the plant organs under study. This is far beyond the scope of this study and, in practice, doing this in a comprehensive manner would be extremely challenging. For instance, it is likely that the equipment required to measure heterogeneity of light, temperature and humidity in the immediate surroundings of a plant organ would affect that heterogeneity itself as it would cast shadows, change air circulation, and so forth. Also, it is far from clear how measurements of heterogeneity would have to be integrated over time to quantify the role of plasticity.

The main conclusion, at this point, is that investigators need to take into account that FA in plants and other sessile organisms originates from a combination of developmental instability and phenotypic plasticity in response to environmental heterogeneity. The relative contributions of these two sources of variation are currently unknown. Motile animals are less affected by this phenomenon because environmental heterogeneities will change in direction and intensity as each individual moves through its environment, and it is thus likely that differences between body sides effectively will average out (Nijhout & Davidowitz, 2003; Klingenberg, 2015). Even for studies of motile animals, however, FA from phenotypic plasticity may be a serious concern if animals are mostly stationary during an important developmental phase, such as the pupal stage in many holometabolous insects (Van Dongen, 2006). This problem is therefore important for many applications of FA in studies of ecology and evolution.

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References

Abeli, T., Zubani, L., Bonomi, C., Parolo, G. & Gargano, D. 2016. Is phenotypic canalization involved in the decline of the endemic *Aquilegia thalictrifolia*? Rethinking relationships between fluctuating asymmetry and species conservation status. *Plant Species Biol.* **31**: 247–255.

Adams, D.C., Rohlf, F.J. & Slice, D.E. 2013. A field comes of age: geometric morphometrics in

512 the 21st century. *Hystrix* **24**: 7–14.

513 Albarrán-Lara, A.L., Mendoza-Cuenca, L., Valencia-Avalos, S., González-Rodríguez, A. &
514 Oyama, K. 2010. Leaf fluctuating asymmetry increases with hybridization and introgression
515 between *Quercus magnoliifolia* and *Quercus resinosa* (Fagaceae) through an altitudinal
516 gradient in Mexico. *Int. J. Plant Sci.* **171**: 310–322.

517 Alves-Silva, E. & Del-Claro, K. 2016. Herbivory-induced stress: leaf developmental instability is
518 caused by herbivore damage in early stages of leaf development. *Ecol. Indic.* **61**: 359–365.

519 Andalo, C., Bazin, A. & Shykoff, J.A. 2000. Is there a genetic basis for fluctuating asymmetry
520 and does it predict fitness in the plant *Lotus corniculatus* grown in different environmental
521 conditions? *Int. J. Plant Sci.* **161**: 213–220.

522 Anton, K.A., R. Ward, J. & Cruzan, M.B. 2013. Pollinator-mediated selection on floral
523 morphology: evidence for transgressive evolution in a derived hybrid lineage. *J. Evol. Biol.*
524 **26**: 660–673.

525 Baranov, S.G. 2014. Use of morphometric method for study fluctuating asymmetry in leaves
526 *Tilia cordata* under industrial pollution . *Adv. Environ. Biol.* **8**: 2391–2398.

527 Baranov, S.G. & Gavrikov, D.E. 2013. Use of TPS software for studying fluctuating asymmetry
528 in flowers. *Int. J. Biosci. Biochem. Bioinforma.* **3**: 284–287.

529 Beasley, D.A.E., Bonisoli-Alquati, A. & Mousseau, T.A. 2013. The use of fluctuating
530 asymmetry as a measure of environmentally induced developmental instability: A meta-
531 analysis. *Ecol. Indic.* **30**: 218–226.

532 Brock, M.T. & Weinig, C. 2007. Plasticity and environment-specific covariances: an

533 investigation of floral–vegetative and within flower correlations. *Evolution* **61**: 2913–2924.

534 Chitwood, D.H., Headland, L.R., Ranjan, A., Martinez, C.C., Braybrook, S.A., Koenig, D.P., *et*
535 *al.* 2012. Leaf asymmetry as a developmental constraint imposed by auxin-dependent
536 phyllotactic patterning. *Plant Cell* **24**: 2318–2327.

537 Cornelissen, T. & Stiling, P. 2005. Perfect is best: low leaf fluctuating asymmetry reduces
538 herbivory by leaf miners. *Oecologia* **142**: 46–56.

539 Cornelissen, T. & Stiling, P. 2010. Small variations over large scales: fluctuating asymmetry
540 over the range of two oak species. *Int. J. Plant Sci.* **171**: 303–309.

541 Cowart, N. & Graham, J. 1999. Within- and among-individual variation in fluctuating
542 asymmetry of leaves in the fig (*Ficus carica* L.). *Int. J. Plant Sci.* **160**: 116–121.

543 Debat, V. 2016. Symmetry is beauty – or is it? Grandeur et décadence de l'asymétrie fluctuante.
544 *Med. Sci.* **32**: 774–780.

545 Dryden, I.L. & Mardia, K. V. 2016. *Statistical shape analysis, with applications in R*, 2nd ed.
546 Wiley, Chichester.

547 Freeman, D.C., Brown, M.L., Dobson, M., Jordan, Y., Kizy, A., Micallef, C., *et al.* 2003.
548 Developmental instability: measures of resistance and resilience using pumpkin (*Cucurbita*
549 *pepo* L.). *Biol. J. Linn. Soc.* **78**: 27–41.

550 Frey, F.M. & Bukoski, M. 2014. Floral symmetry is associated with flower size and pollen
551 production but not insect visitation rates in *Geranium robertianum* (Geraniaceae). *Plant*
552 *Species Biol.* **29**: 272–280.

553 Gajić, M. 1983. The flora of the Deliblato Sand. *Fac. Nat. Sci. Inst. Biol. Univ. Novi Sad, Novi*

554 *Sad, Serbia* 6–446.

555 Gardner, A.G., Gerald, J.N.F., Menz, J., Shepherd, K.A., Howarth, G. & Jabaily, R.S. 2016.
556 Characterizing floral symmetry in the core Goodeniaceae with geometric morphometrics.
557 *PLoS One* **11**: e0154736.

558 Graham, J.H., Raz, S., Hel-Or, H. & Nevo, E. 2010. Fluctuating asymmetry: methods, theory,
559 and applications. *Symmetry (Basel)*. **2**: 466–540.

560 Helsen, P. & Van Dongen, S. 2016. Associations between floral asymmetry and individual
561 genetic variability differ among three prockly pear (*Opuntia echios*) populations. *Symmetry*
562 *(Basel)*. **8**: 116.

563 Herrera, C.M. 2009. *Multiplicity in unity: plant subindividual variation and interactions with*
564 *animals*. University of Chicago Press, Chicago.

565 Houle, D. 1998. High enthusiasm and low R-squared. *Evolution (N. Y)*. **52**: 1872–1876.

566 Ivanović, A. & Kalezić, M.L. 2010. Testing the hypothesis of morphological integration on a
567 skull of a vertebrate with a biphasic life cycle: a case study of the alpine newt. *J. Exp. Zool.*
568 *Part B Mol. Dev. Evol.* **314**: 527–538.

569 Jamniczky, H.A. & Hallgrímsson, B. 2011. Modularity in the skull and cranial vasculature of
570 laboratory mice: implications for the evolution of complex phenotypes. *Evol. Dev.* **13**: 28–
571 37.

572 Klingenberg, C.P. 2003a. A developmental perspective on developmental instability: theory,
573 models and mechanisms. In: *Developmental instability: causes and consequences* (M.
574 Polak, ed), pp. 14–34. Oxford University Press, New York.

Klingenberg, C.P. 2015. Analyzing fluctuating asymmetry with geometric morphometrics: concepts, methods, and applications. *Symmetry (Basel)*. **7**: 843–934.

Klingenberg, C.P. 2003b. Developmental instability as a research tool: using patterns of fluctuating asymmetry to infer the developmental origins of morphological integration. In: *Developmental instability: causes and consequences* (M. Polak, ed), pp. 427–442. Oxford University Press, New York.

Klingenberg, C.P. 2010. Evolution and development of shape: integrating quantitative approaches. *Nat. Rev. Genet.* **11**: 623–635.

Klingenberg, C.P. 2011. MorphoJ: an integrated software package for geometric morphometrics. *Mol. Ecol. Resour.* **11**: 353–357.

Klingenberg, C.P. 2013. Visualizations in geometric morphometrics: How to read and how to make graphs showing shape changes. *Hystrix* **24**: 15–24.

Klingenberg, C.P., Barluenga, M. & Meyer, A. 2002. Shape analysis of symmetric structures: quantifying variation among individuals and asymmetry. *Evolution (N. Y).* **56**: 1909–1920.

Klingenberg, C.P., Duttke, S., Whelan, S. & Kim, M. 2012. Developmental plasticity, morphological variation and evolvability: a multilevel analysis of morphometric integration in the shape of compound leaves. *J. Evol. Biol.* **25**: 115–129.

Klingenberg, C.P. & Nijhout, H.F. 1999. Genetics of fluctuating asymmetry: a developmental model of developmental instability. *Evolution (N. Y).* **53**: 358–375.

Koethe, S., Bloemer, J. & Lunau, K. 2017. Testing the influence of gravity on flower symmetry in five *Saxifraga* species. *Naturwissenschaften* **104**: 37.

596 Komac, B. & Alados, C.L. 2012. Fluctuating asymmetry and *Echinospartum horridum* fitness
597 components. *Ecol. Indic.* **18**: 252–258.

598 Kozlov, M. V, Wilsey, B.J., Koricheva, J. & Haukioja, E. 1996. Fluctuating asymmetry of birch
599 leaves increases under pollution impact. *J. Appl. Ecol.* **33**: 1489–1495.

600 Kubínová, Z., Janáček, J., Lhotáková, Z. & Šprtová, M. 2017. Norway spruce needle size and
601 cross section shape variability induced by irradiance on a macro- and microscale and -.
602 *Trees* Advance online, DOI: 10.1007/s00468-017-1626-3.

603 Kurepin, L. V, Yeung, E.C., Reid, D.M. & Pharis, R.P. 2016. Light signaling regulates tulip
604 organ growth and ethylene production in a tissue-specific manner. *Int. J. Plant Sci.* **177**:
605 339–346.

606 Labonne, G., Navarro, N., Laffont, R., Chateau-Smith, C. & Montuire, S. 2014. Developmental
607 integration in a functional unit: deciphering processes from adult dental morphology. *Evol.*
608 *Dev.* **16**: 224–232.

609 Larcher, W. 2003. *Physiological Plant Ecology*, 4th ed. Springer-Verlag, Berlin.

610 Leamy, L.J. & Klingenberg, C.P. 2005. The genetics and evolution of fluctuating asymmetry.
611 *Annual Review of Ecology, Evolution and Systematics* **3**6: 1–21.

612 Lens, L., Van Dongen, S., Kark, S. & Matthysen, E. 2002. Fluctuating asymmetry as an indicator
613 of fitness: can we bridge the gap between studies? *Biol. Rev.* **77**: 27–38.

614 Maffei, M.E. 2014. Magnetic field effects on plant growth, development, and evolution. *Front.*
615 *Plant Sci.* **5**: 445.

616 Manitašević Jovanović, S., Tucić, B. & Matić, G. 2011. Differential expression of heat-shock

proteins Hsp70 and Hsp90 in vegetative and reproductive tissues of *Iris pumila*. *Acta Physiol. Plant.* **33**: 233–240.

Martinez, C.C., Chitwood, D.H., Smith, R.S. & Sinha, N.R. 2016. Left–right leaf asymmetry in decussate and distichous phyllotactic systems. *Philos. Trans. R. Soc. B Biol. Sci.* **371**: 20150412.

Mathew, B. 1981. *The Iris*. Timber Press, Portland, OR.

Miljković, D. 2012. Developmental stability of *Iris pumila* flower traits: A common garden experiment. *Arch. Biol. Sci.* **64**: 123–133.

Møller, A.P. 1995. Bumblebee preference for symmetrical flowers. *Proc. Natl. Acad. Sci. U. S. A.* **92**: 2288–2292.

Møller, A.P. & Shykoff, J.A. 1999. Morphological developmental statibility in plants: patterns and causes. *Int. J. Plant Sci.* **160**: S135–S146.

Nijhout, H.F. & Davidowitz, G. 2003. Developmental perspectives on phenotypic variation, canalization, and fluctuating asymmetry. In: *Developmental instability: causes and consequences* (M. Polak, ed), pp. 3–13. Oxford University Press: New York, NY, USA.

Palmer, A.R. 1996. Waltzing with asymmetry. *Bioscience* 518–532.

Palmer, A.R. & Hammond, L.M. 2000. The emperor's codpiece: a post-modern perspective on biological asymmetries. *Int. Soc. Behav. Ecol. Newsl.* **12**: 13–20.

Palmer, A.R. & Strobeck, C. 1986. Fluctuating asymmetry: measurement, analysis, patterns. *Annu. Rev. Ecol. Syst.* 391–421.

Palmer, A.R. & Strobeck, C. 2003. Fluctuating asymmetry analyses revisited. In: *Developmental*

Instability: Causes and Consequences (M. Polak, ed), pp. 279–319. Oxford University Press, New York.

Pande, P.C. & Singh, V. 1981. Floral development of *Iris decora* Wall. (Iridaceae). *Bot. J. Linn. Soc.* **83**: 41–56.

Parsons, P.A. 1992. Fluctuating asymmetry: a biological monitor of environmental and genomic stress. *Heredity (Edinb).* **68**: 361–364.

Pélabon, C., Hansen, T.F., Carlson, M.L. & Armbruster, W.S. 2006. Patterns of asymmetry in the twining vine *Dalechampia scandens* (Euphorbiaceae): ontogenetic and hierarchical perspectives. *New Phytol.* **170**: 65–74.

Perfectti, F. & Camacho, J.P.M. 1999. Analysis of genotypic differences in developmental stability in *Anona cherimola*. *Evolution (N. Y).* **53**: 1396–1405.

Polak, M. 2003. *Developmental instability: causes and consequences*. Oxford University Press.

Radović, S., Urošević, A., Hočevar, K., Vuleta, A., Manitašević Jovanović, S. & Tucić, B. 2017. Geometric morphometrics of functionally distinct floral organs in *Iris pumila*. *Arch. Biol. Sci.* **69**: 223–231.

Randolph, L.F. 1955. The geographic distribution of European and eastern Mediterranean species of bearded *Iris*. In: *Iris Year Book*, pp. 35–46.

Rao, G.-Y., Andersson, S. & Widén, B. 2002. Flower and cotyledon asymmetry in *Brassica cretica*: genetic variation and relationships with fitness. *Evolution (N. Y).* **56**: 690–698.

Raz, S., Graham, J.H., Hel-Or, H., Pavlíček, T. & Nevo, E. 2011. Developmental instability of vascular plants in contrasting microclimates at "Evolution Canyon." *Biol. J. Linn. Soc.* **102**:

786–797.

Rohlf, F.J. 2006. tpsDig, version 2.10. *Dep. Ecol. Evol. State Univ. New York, Stony Brook*.

Sandner, T.M. & Matthies, D. 2017. Fluctuating asymmetry of leaves is a poor indicator of environmental stress and genetic stress by inbreeding in *Silene vulgaris*. *Ecol. Indic.* **79**: 247–253. Elsevier.

SAS Institute Inc. 1990. *SAS STAT User's Guide*. SAS Institute Inc., Cary, NC.

Savriama, Y., Gómez, J.M., Perfectti, F. & Klingenberg, C.P. 2012. Geometric morphometrics of corolla shape: dissecting components of symmetric and asymmetric variation in *Erysimum mediohispanicum* (Brassicaceae). *New Phytol.* **196**: 945–954.

Savriama, Y. & Klingenberg, C. 2011. Beyond bilateral symmetry: geometric morphometric methods for any type of symmetry. *BMC Evol. Biol.* **11**: 280.

Schoute, J.C. 1935. On corolla aestivation and phyllotaxis of floral phyllomes. *Verh. der K. Akad. van Wet. te Amsterdam, Afd. Natuurkd.* **34**: 1–77.

Siikamäki, P. & Lammi, A. 1998. Fluctuating asymmetry in central and marginal populations of *Lychnis viscaria* in relation to genetic and environmental factors. *Evolution (N. Y).* **52**: 1285–1292.

Simmons, L.W., Tomkins, J.L., Kotiaho, J.S. & Hunt, J. 1999. Fluctuating paradigm. *Proc. R. Soc. London B, Biol. Sci.* **266**: 593–595.

Taiz, L. & Zeiger, E. 2010. *Plant Physiology*, 5th ed. Sinauer Associates, Sunderland, MA.

Telhado, C., Silveira, F.A.O., Fernandes, G.W. & Cornelissen, T. 2017. Fluctuating asymmetry in leaves and flowers of sympatric species in a tropical montane environment. *Plant Species*

680 *Biol.* **32**: 3–12.

681 Tucić, B., Manitašević, S., Vuleta, a. & Matić, G. 2008. Linking Hsp90 function to micro-
682 environmental and stochastic variation in floral organs of *Iris pumila* L. *Arch. Biol. Sci.* **60**:
683 411–419.

684 Tucić, B. & Miljković, D. 2010. Fluctuating asymmetry of floral organ traits in natural
685 populations of *Iris pumila* from contrasting light habitats. *Plant Species Biol.* **25**: 173–184.

686 Tucić, B., Milojković, S., Vujčić, S. & Tarasjev, A. 1988. Clonal diversity and dispersion in *Iris*
687 *pumila*. *Acta oecologica. Oecologia Plant.* **9**: 211–219.

688 Tucić, B., Vuleta, A. & Manitašević-Jovanović, S. 2013. Exploring phenotypic floral integration
689 in *Iris pumila* L.: A common-garden experiment. *Arch. Biol. Sci.* **65**: 781–794.

690 Van Dongen, S. 2006. Fluctuating asymmetry and developmental instability in evolutionary
691 biology: past, present and future. *J. Evol. Biol.* **19**: 1727–1743.

692 Vaupel, A. & Matthies, D. 2012. Abundance, reproduction, and seed predation of an alpine plant
693 decrease from the center toward the range limit. *Ecology* **93**: 2253–2262.

694 Waldmann, P. 2001. The effect of inbreeding on fluctuating asymmetry in *Scabiosa canescens*
695 (Dipsacaceae). *Evol. Ecol.* **15**: 117–127.

696 Weinig, C. 2002. Phytochrome photoreceptors mediate plasticity to light quality in flowers of the
697 Brassicaceae. *Am. J. Bot.* **89**: 230–235.

698 Wilsey, B.J., Haukioja, E., Koricheva, J. & Sulkinoja, M. 1998. Leaf fluctuating asymmetry
699 increases with hybridization and elevation in tree-line birches. *Ecology* **79**: 2092–2099.

700 Zelditch, M.L., Swiderski, D.L. & Sheets, H.D. 2012. *Geometric morphometrics for biologists: a*

primer, 2nd ed. Academic Press, London.

Zelditch, M.L., Wood, A.R. & Swiderski, D.L. 2009. Building developmental integration into functional systems: function-induced integration of mandibular shape. *Evol. Biol.* **36**: 71–87.

706 Table1. Size of floral organs in response to orientation. Tabled values are the sample size (*N*), the mean centroid size and its standard error (SE).

	Fall			Standard			Style branch		
Orientation	*N*	Mean	SE	*N*	Mean	SE	*N*	Mean	SE
0˚	266	7.375	0.211	262	7.433	0.207	257	6.865	0.162
60˚	265	7.354	0.206	262	7.394	0.216	257	6.871	0.157
120˚	266	7.344	0.214	262	7.402	0.210	257	6.868	0.158
180˚	266	7.360	0.218	262	7.399	0.217	257	6.874	0.157
240˚	266	7.344	0.217	262	7.394	0.208	257	6.872	0.154
300˚	266	7.350	0.209	262	7.391	0.217	257	6.869	0.157

707 Table 2. Decomposition of Procrustes sums of squares for the different flower parts (using an
708 expanded version of formula 2 in Savriama & Klingenberg, 2011). For each flower part, the
709 decomposition has been done separately for the symmetric and asymmetry components of
710 shape variation, and both have been combined to quantify the total shape variation. The
711 percentages indicate the proportions of asymmetry within flowers for which orientation can
712 and cannot account.

	Fall	Standard	Style branch
Symmetric component of part shape variation			
Orientation	0.1415	0.2147	0.0059
	(11.5%)	(12.8%)	(1.6%)
Plant	4.0045	4.6509	2.3826
Flower	0.7545	0.9263	0.3683
Other asymmetry	1.0851	1.4601	0.3643
	(88.5%)	(87.2%)	(98.4%)
Total	5.9856	7.2520	3.1212
Asymmetric component of part shape variation			
Orientation	0.0157	0.0117	0.0073
	(5.7%)	(7.3%)	(3.1%)
Plant	0.0811	0.0939	0.0566
Flower	0.0534	0.0713	0.0505
Other asymmetry	0.2617	0.3016	0.2308
	(94.3%)	(96.3%)	(96.9%)
Total	0.4118	0.4785	0.3452
Total shape variation (symmetric and asymmetry components combined)			

Orientation	0.1571	0.2263	0.0132
	(10.4%)	(11.4%)	(2.2%)
Plant	4.0856	4.7448	2.4392
Flower	0.8079	0.9976	0.4189
Other asymmetry	1.3468	1.7618	0.5950
	(89.6%)	(88.6%)	(97.8%)
Total	6.3974	7.7305	3.4663

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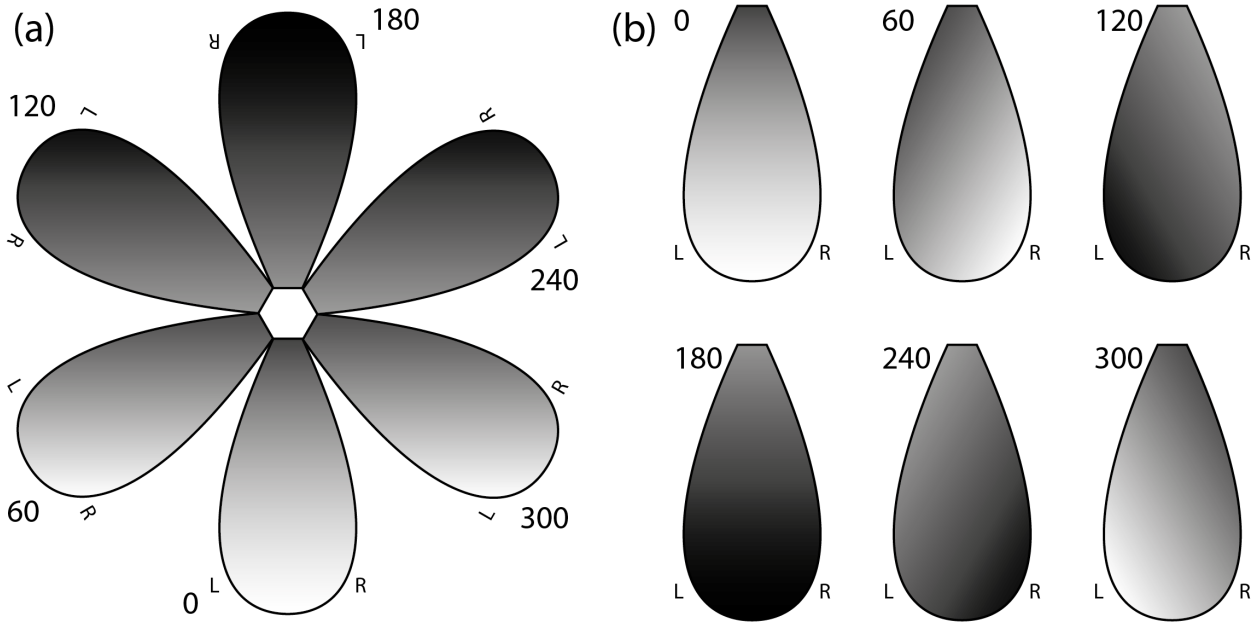
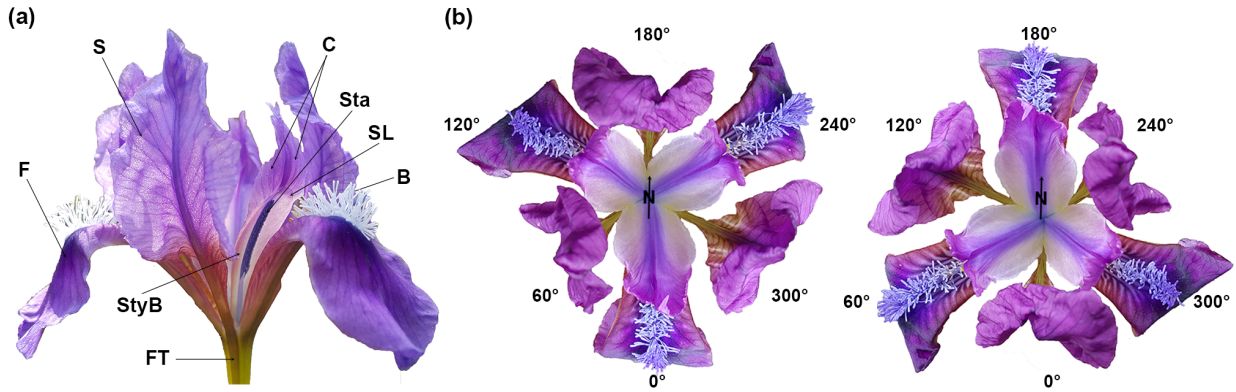
714



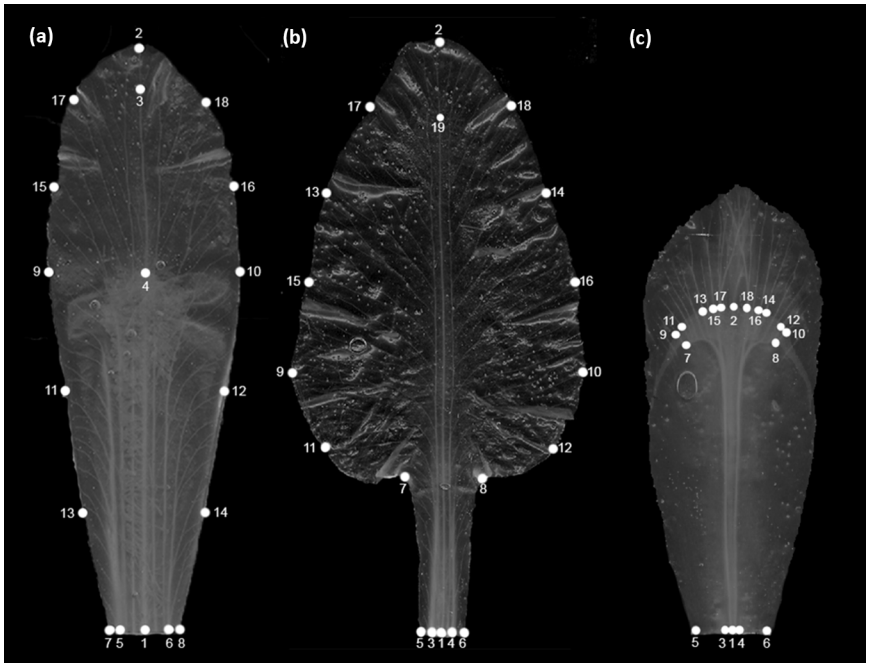
715

716 **Figure 1.** Effects of an environmental gradient on plant parts with different orientations. (A)
717 Plant parts in their natural arrangement. The environmental gradient acts in a vertical
718 direction from the bottom of the diagram (0˚) to the top (180˚) and is represented by a
719 gradation from light to dark shading. As a result of the different orientation of the parts, the
720 anatomical axes of each part appear at a different angle to the gradient (L and R mark the left
721 and right sides of each part). (B) The effects of the gradient in relation to the parts viewed
722 separately. Parts have been rearranged to have the same orientation in relation to their
723 anatomical axes. As a consequence, the effects of the gradient are in directions that are
724 distinctive for each one of the parts. If there is phenotypic plasticity in response to the
725 environmental gradient, the resulting morphological differences may also be specific
726 according to the orientation of parts. Note that this argument does not depend on the number
727 or particular arrangement of parts. In conventional studies that do not specifically record the
728 compass orientation of the plant parts under study, differences due to phenotypic plasticity in
729 response to such a gradient would be considered as fluctuating asymmetry.



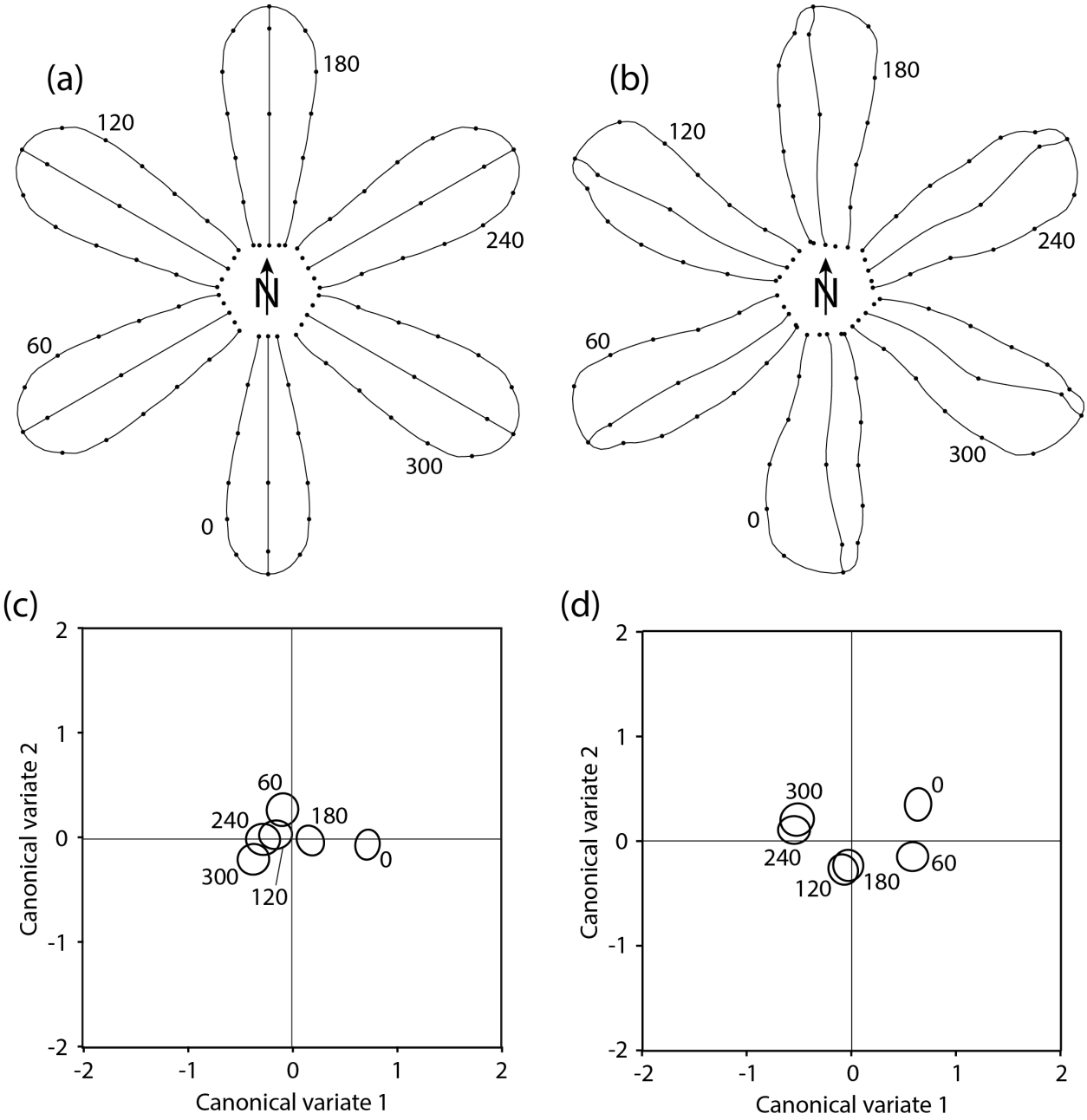
730

731 **Figure 2.** Representative photograph of an *Iris pumila* flower. (A). Side-view image of an
732 *Iris pumila* flower, with acronyms of floral organs and their corresponding parts (according
733 to Mathew 1981): F- fall, S-standard, StyB- style branch, C-crest, Sta- stamen, SL-stigmatic
734 lip, B-beard, FT- floral tube; (B). Top view of an *Iris pumila* flower and six orientations of
735 floral organs (0º toward the Sun).



736

737 **Figure 3.** Configuration of landmarks on the images of floral organs: (A) fall; (B) standard
738 and (C) style branch.



739

740 **Figure 4.** Effects of orientation on the shape of the falls. (A). Differences among the six
741 orientations of falls in the means of the symmetric component of shape variation (shape
742 changes exaggerated five-fold); (B). Differences among the six orientations in the means of
743 the asymmetric component of shape variation (shape changes exaggerated 15-fold); (C). 95%
744 confidence ellipses for the means of the symmetric component of shape variation in the six
745 orientations; (D). 95% confidence ellipses for the means of the asymmetry component of
746 shape variation in the six orientations.

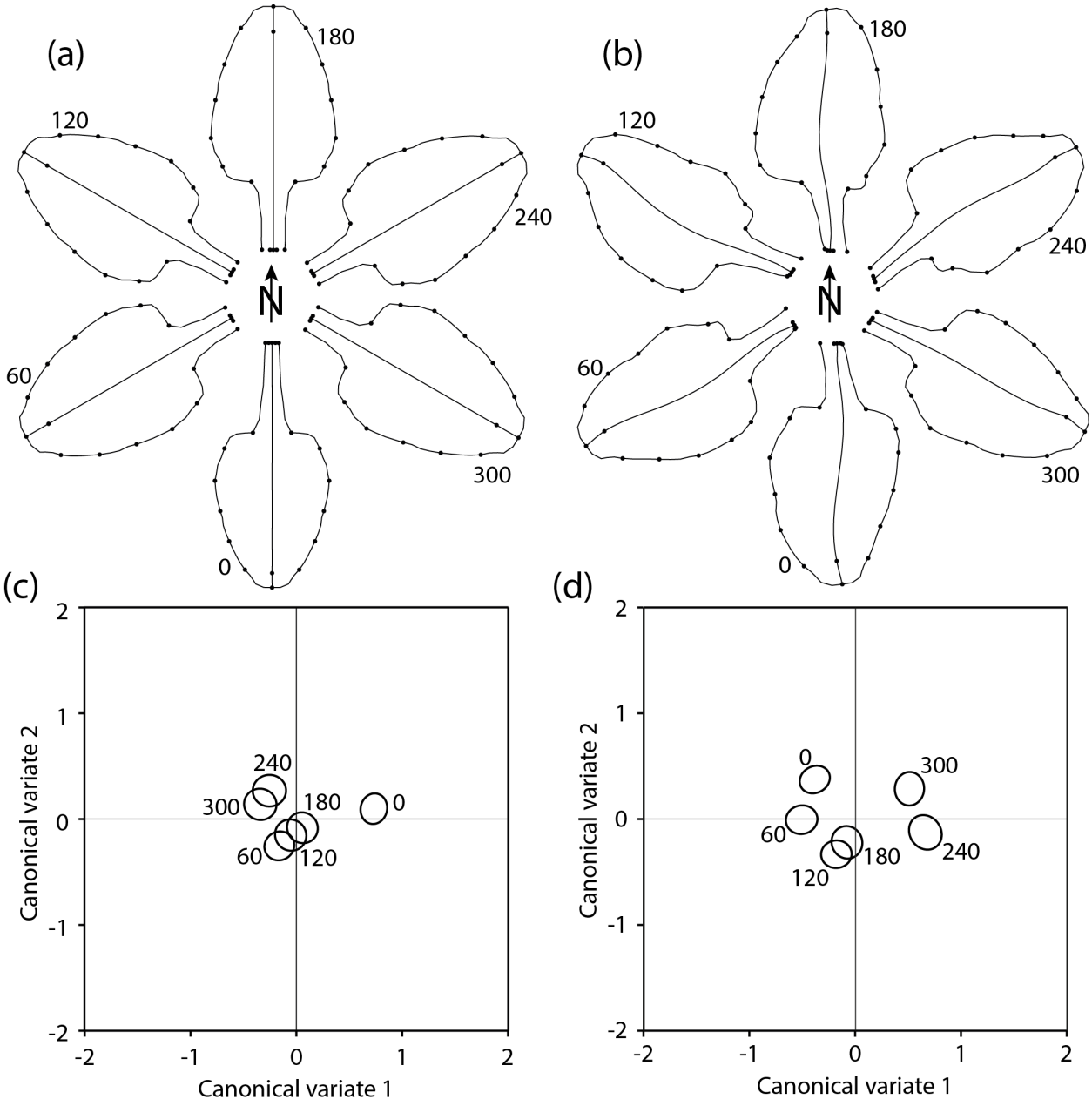


Figure 5. Effects of orientation on the shape of the standards. (A). Differences among the six orientations of standards in the means of the symmetric component of shape variation (shape changes exaggerated five-fold); (B). Differences among the six orientations of standards in the means of the asymmetric component of shape variation (shape changes exaggerated 15-fold); (C). 95% confidence ellipses for the means of the symmetric component of shape variation in the six orientations; (D). 95% confidence ellipses for the means of the asymmetry component of shape variation in the six orientations.

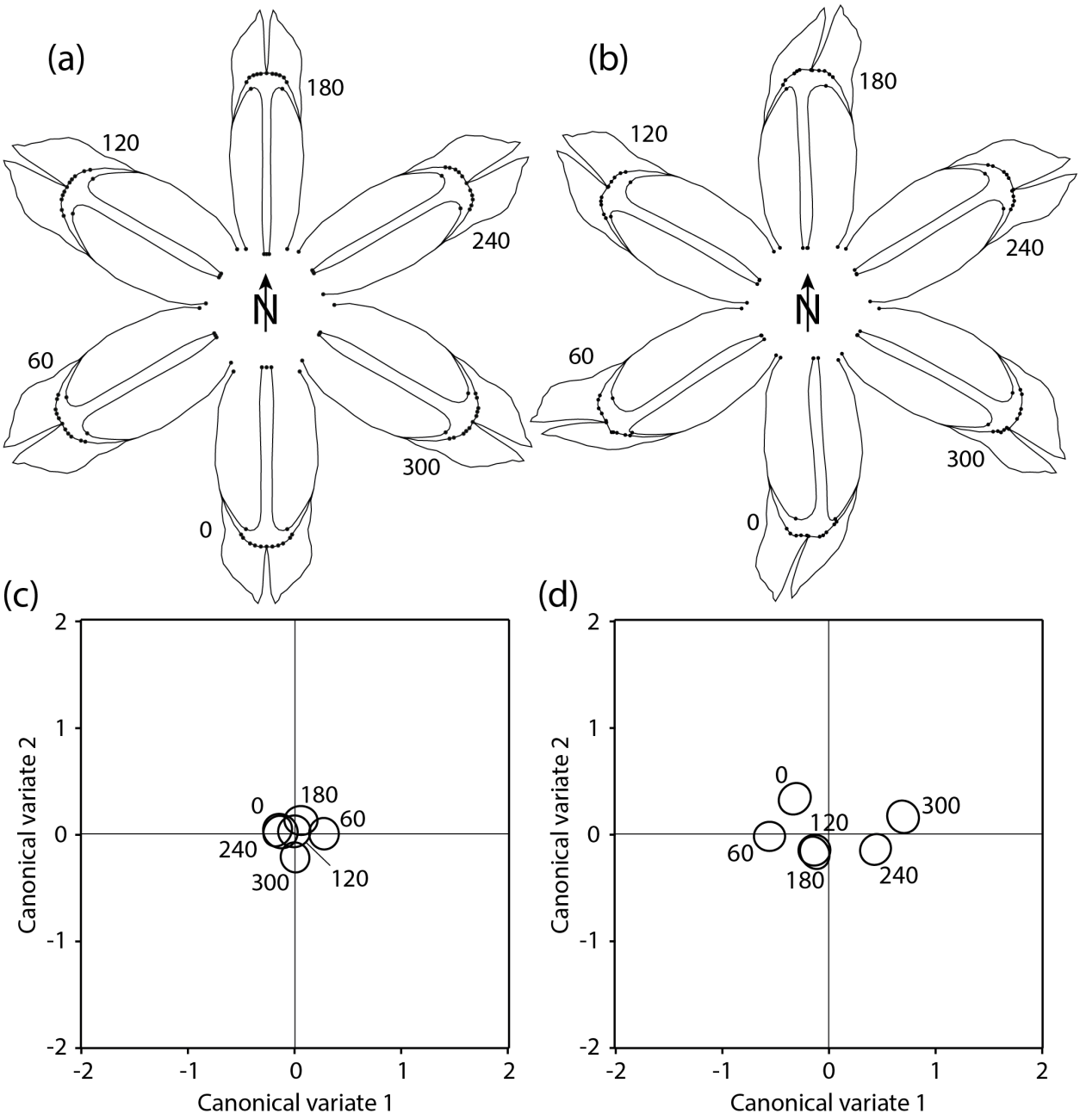


Figure 6. Effects of orientation on the shape of the style branches. (A). Differences among the six orientations of style branches in the means of the symmetric component of shape variation (shape changes exaggerated 15-fold); (B). Differences among the six orientations in the means of the asymmetric component of shape variation (shape changes exaggerated 15-fold). Note that there are no landmarks on the terminal lobes—the shape changes in this region are extrapolated from the nearby landmarks on the stigmatic lip; (C). 95% confidence ellipses for the means of the symmetric component of shape variation in the six orientations;

763 (D). 95% confidence ellipses for the means of the asymmetry component of shape variation

764 in the six orientations.